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Oxaliplatin-Induced Neuropathy: Oxidative Stress as Pathological Mechanism. Protective Effect of Silibinin

Lorenzo Di Cesare Mannelli, Matteo Zanardelli, Paola Failli, and Carla Ghelardini
Deparment of Preclinical and Clinical Pharmacology, University of Florence, Florence, Italy.

Abstract: Oxaliplatin is the standard treatment for advanced colorectal cancer. Its dose-limiting toxicity is the development of a painful neuropathic syndrome sustained by unclear mechanisms. Although the oxidative hypothesis is a matter of debate, direct data about oxidative damage induced in vivo by anticancer agents are lacking and the efficacy of the available antioxidant compounds are unsatisfactory. In a rat model of painful oxaliplatin-induced neuropathy (2.4 mgkg^{-1} i.p., daily for 21 days), we described an important component of oxidative stress. In the plasma of oxaliplatin-treated rats, the increases in carbonylated protein and thiobarbituric acid reactive substances were the index of the resultant protein oxidation and lipoperoxidation, respectively. The same pattern of oxidation was revealed also in the sciatic nerve, and in the spinal cord where the damage reached the DNA level. The antioxidant compound silibinin (100 mgkg^{-1} per os), administered once a day, starting from the first day of oxaliplatin injection until the 20th, prevented oxidative damage as did α-tocopherol. Repetitive administration of silibinin, as well as α-tocopherol, reduced oxaliplatin-dependent pain induced by mechanical and thermal stimuli. Antioxidants were also able to improve motor coordination. The antineuropathic effect of both molecules improved by about 50% oxaliplatin-induced behavioral alterations.

***Perspective:** This study characterizes oxidative stress parameters in a rat model of oxaliplatin-induced neuropathy. A relationship between the improvement of oxidative alterations and pain relief is established in rats treated with natural antioxidant compounds like α-tocopherol and silibinin. Silibinin could be a valid therapeutic option for chemotherapy-induced neuropathy.*

***Key words:** Neuropathic pain, α-tocopherol, disease modifying agent, lipoperoxidation, carbonylated protein.*

Oxaliplatin is a platin-organic drug with antineoplastic properties electively used for the treatment of advanced colorectal cancer and a valid option for patients in the adjuvant setting. Unlike other platinum derivatives, its use induces only mild hematological and gastrointestinal side effects, and does not result in significant renal impairment or ototoxicity. The dose-limiting toxicity of oxaliplatin is the development of a neuropathic syndrome with paresthesia, dysesthesia, and pain. A tangled panel of symptoms may be disabling for treated patients, adversely affecting activities of daily living and thereby quality of life, until suspension of the therapy.[12,17,42] Whereas neuropathy acutely developed is usually self-limiting, often resolving the symptoms within days, repeated oxaliplatin administration induces a chronic neuropathy that cannot be resolved between cycles.[3,9,14,17]

Randomized trials demonstrating a prophylactic or therapeutic effect of antihyperalgesic drugs on oxaliplatin's cumulative neurotoxicity are still lacking or inconclusive.[2,24] Among pharmacological treatments, antioxidant compounds have been tested as possible therapeutic approaches. In particular, glutathione, N-acetylcysteine, and vitamin E (α-tocopherol) may have a clinical role in patient neuroprotection.[4,6,7,25,29,35] However, the major bias of these clinical trials resides in their small size and/or in their short term follow-up.[2]

An important limit in this field is the insufficient information on the molecular basis of the neuropathy. Despite the empirical therapeutic use of antioxidants, the oxidative hypothesis in oxaliplatin neurotoxicity is still a matter of debate. In mouse hybrid neurons, Park

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Address reprint requests to Lorenzo Di Cesare Mannelli, Dept of Preclinical and Clinical Pharmacology, University of Florence, Viale Pieraccini 6, 50139, Florence, Italy. E-mail: lorenzo.mannelli@unifi.it
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et al[37] suggest that reactive oxygen species (ROS) by activation of p53 signaling play a major role in cisplatin-induced neuronal apoptotic cell death. Antioxidants as N-acetylcysteine can reduce this apoptotic pathway attenuating p53 accumulation.[37] Moreover, glutathione-inhibitable production of superoxide anion ($O_2^{-\cdot}$) has been described for oxaliplatin in CHO cells.[34]

As things stand today, direct data about oxidative damage induced in vivo by platin derivatives are however still lacking. Moreover, the absence of a clear oxidation target hampers the finding of more active antioxidant compounds.

In order to directly evaluate the role of oxidative stress in oxaliplatin neurotoxicity, we characterized the oxidative profile of plasma and the nervous system in a rat model of oxaliplatin-induced neuropathy. To study the relationship between oxidative status and pain, a repeated treatment with the natural antioxidant compound α-tocopherol was performed. Aimed to pinpoint new potential antineuropathic treatments, we evaluated the antihyperalgesic effect of silibinin, the principal component of the silymarin complex, an active extract from the seeds of the plant milk thistle (*Silybum marianum*). It has been shown that silibinin inhibits overproduction of $O_2^{-\cdot}$ by stimulated neutrophils, xanthine oxidase activity, and prevents hem-mediated oxidative modification of low density lipoproteins.[39,42]

Methods

Animals

For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy) weighing approximately 200 to 250 g at the beginning of the experimental procedure were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Four rats were housed per cage (size 26 $\times$ 41 cm); animals were fed with standard laboratory diet and tap water ad libitum, and kept at 23 $\pm$ 1°C with a 12 hour light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Animals were anesthetized before cervical dislocation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Oxaliplatin Model

Rats were treated with 2.4 mgkg^{-1} oxaliplatin, administered intraperitoneally (i.p.) for 5 consecutive days every week for 3 weeks (15 i.p. injections).[8] Oxaliplatin was dissolved in 5% glucose solution. Control animals received an equivalent volume of 5% glucose i.p. Behavioral and biochemical tests were performed at the 21st day. The time course of behavioral measures revealed that pain began on day 14 but not uniformly in all animals.

Drug Treatments

Silibinin (Sigma-Aldrich, Milan, Italy) and α-tocopherol (Sigma-Aldrich, Milan, Italy) were used at 100 mgkg^{-1}. Both drugs were suspended in 1% carboxymethylcellulose sodium salt (CMC) and administered by the per os (p.o.) route. Repeated treatment (chronic) consisted of a daily administration following the same protocol described for oxaliplatin from the first up to the 20th day. Behavioral and biochemical tests were performed 24 hours after the end of treatments. Acute treatment consisted in a single p.o. administration of silibinin and α-tocopherol (100 mgkg^{-1}) at the 21st day of oxaliplatin injection. Control animals received an equivalent volume of 1% CMC p.o.

Paw Pressure Test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al.[27] Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (25%). For analgesia measures, mechanical pressure application was stopped at 120 g. Experiments were performed blind.

Von Frey Test

The animals were placed in 20- $\times$ 20-cm plexiglas boxes equipped with a metallic meshy floor, 20 cm above the bench. A habituation of 15 minutes was allowed before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: the withdrawal threshold was evaluated by applying force ranging from 0 to 50 grams with a .2 gram accuracy. Punctuate stimulus was delivered to the midplantar area of each anterior paw from below the meshy floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. Paw sensitivity threshold was defined as the minimum pressure required to elicit a robust and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not taken as a withdrawal response. Stimuli were applied on each anterior paw with an interval of 5 seconds. The measure was repeated 5 times and the final value was obtained by averaging the 5 measures.[43]

Cold Plate Test

The animals were placed on a stainless box (12- $\times$ 20- $\times$ 10-cm) with a cold plate as floor. The temperature of the

cold plate was kept constant at 4 ± 1°C. Pain-related behaviors (ie, lifting and licking of the hind paw) were observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 seconds.

Rota-Rod Test

Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a nonslippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed on the basis of walking time and the number of falls from the rod for a maximum of 10 minutes (600 seconds). After a maximum of 6 falls from the rod, test was suspended and the time was recorded.

Tissue Processing

After behavioral tests, animals were sacrificed, blood was collected in heparin-treated tubes, and plasma fraction was isolated by centrifugation. The sciatic nerve and the lumbar portion of spinal cord were isolated, immediately frosted in liquid nitrogen, and fragmented. Part of the obtained powder was treated with TRI-Reagent (Sigma-Aldrich, Milan, Italy) and processed for DNA extraction. The remaining part was homogenized in lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, .5% Triton X-100, Complete Protease Inhibitor (Roche), and the homogenate was incubated on ice for 30 minutes. Then, the suspension was sonicated on ice using 3, 10-second bursts at high intensity with a 10-second cooling period between each burst. The obtained material was used for lipid peroxidation assay and, after centrifugation (13,000 *g* for 15 minutes at 4°C), for Western blot analysis.

Lipid Peroxidation (Thiobarbituric Acid Reactive Substances, TBARS)

Thiobarbituric acid reactive substances (TBARS) were quantified in plasma and tissue homogenates as described previously, with some minor modifications.[36]

100 μl of plasma or 100 μg of tissue homogenate were added to 4 mL reaction mixture consisting of 36 mM thiobarbituric acid (Sigma-Aldrich, Milan, Italy) solubilized in 10% CH_3COOH, .2 % SDS, pH was adjusted to 4.0 with NaOH. The mixture was heated for 60 minutes at 100°C and the reaction was stopped by placing the vials in ice bath for 10 minutes. After centrifugation (at 1,600 *g* at 4°C for 10 minutes) the absorbance of the supernatant was measured at 532 nm (Perkin-Elmer spectrometer, Monza, Italy) and TBARS were quantified in μmoles/milligram of total protein using 1,1,3,3-tetramethoxypropane as standard. Protein homogenate concentration was measured by bicinchoninc acid (BCA; Sigma-Aldrich, Milan, Italy) assay.

Carbonylated Protein Evaluation

Carbonylated proteins were evaluated in tissue homogenates (see previous paragraph) and plasma.

Plasma or nervous tissue proteins extract were quantified by BCA. Twenty μg of each sample were denatured by 6% SDS and derivatized by 15-minute incubation with 2-4 dinitrophenyl hydrazine (DNPH; Sigma-Aldrich, Italy) at room temperature. Samples were separated on a 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis and transferred onto nitrocellulose membranes (Biorad, Italy). Membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing .1% Tween 20 (PBST) and then probed overnight with primary antibody specific versus DNPH (Sigma-Aldrich, Italy) 1:5000 in PBST/5% nonfat dry milk. After washing with PBST, the membranes were incubated for 1 hour in PBST containing the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000; Cell Signalling, USA) and again washed. ECL (Pierce, USA) was used to visualize the peroxidase-coated bands. Densitometric analysis was performed using the "Scion Image" analysis software. For each experiment the density of all bands showed in a lane was reported as mean. For plasma samples Ponceau-stained membranes were used as loading control. β-actin normalization was performed for nervous tissue samples.[13]

DNA Oxidation

One hundred μg DNA for each sample were digested for 2 hours (37°C) in 25 mM CH_3COONa buffer, pH 4.8, containing 1 mM $ZnCl_2$ and 1.1 units of nuclease P1 (Sigma-Aldrich, Milan, Italy). pH was then adjusted to 8.0 using Tris 1.5 M. One unit of alkaline phosphatase (Sigma-Aldrich, Italy) was added and incubated at 37°C for 30 minutes. Samples were boiled for 10 minutes and 8-OH-dG levels were measured in a volume containing 30 μg of initial DNA by ELISA assay (Cayman, Ann Arbor, MI). Measures were performed in triplicate. The absorbance was measured at 420 nm (Perkin-Elmer spectrometer, Monza, Italy) according to manufacturing instructions. The level of DNA oxidation was expressed as pg/mL of 8-OH-dG.

Statistic Analysis

Results were expressed as means ± S.E.M. and the analysis of variance was performed by analysis of variance. A Bonferroni's significant difference procedure was used as post hoc comparison. *P* values of less than .05 or .01 were considered significant. Data were analyzed using the "Origin 8.1" software.

Results

Intraperitoneal daily administration of oxaliplatin (2.4 $mgkg^{-1}$) induced a neuropathy characterized by pain starting from the 14th day (data not shown). On the 21st day, the response to a noxious mechanical stimulus was increased and reached the maximum: the pressure tolerated on the posterior paw, measured by

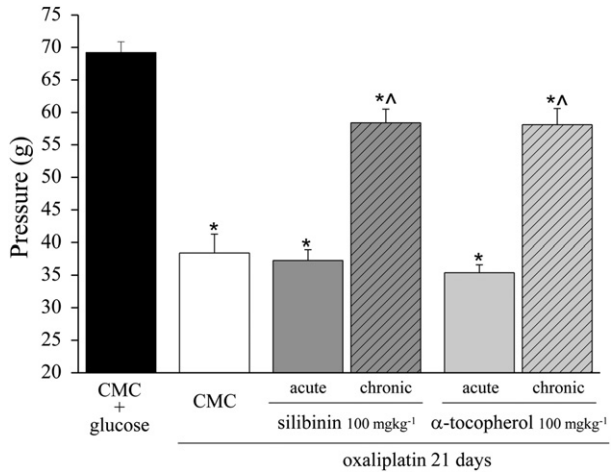

Figure 1. Pain: noxious stimulus, Paw-pressure test. Rats were daily intraperitoneally treated with 2.4 mgkg^{-1} oxaliplatin (dissolved in 5% glucose). Hyperalgesia was evaluated at day 21 by Paw-pressure test. Silibinin or α-tocopherol (100 mgkg^{-1}, dissolved in CMC) were per os administered acutely (single injection 30 minutes before the test; acute) or repetitively (daily for 20 days starting from the first day of oxaliplatin administration; chronic). Control animals were treated with vehicles. Each value represents the mean of 24 rats per group, performed in 2 different experimental sets. *P < .01 versus CMC + glucose (control). ^P < .01 versus CMC-oxaliplatin.

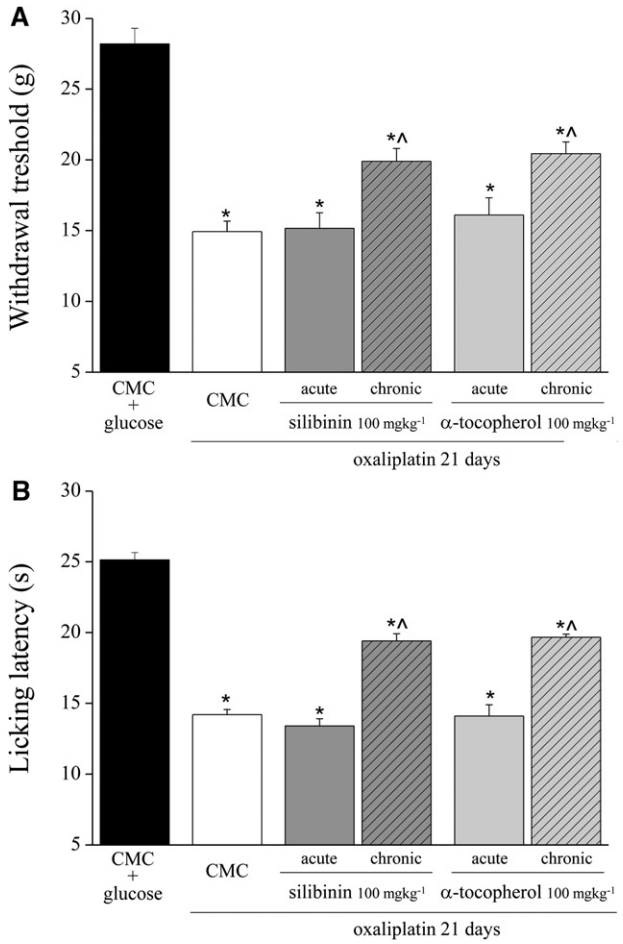

Figure 2. Pain: non-noxious stimuli. **(A)** Von Frey test was used to measure the response evoked by a mechanical stimulus. **(B)** The response to a thermal stimulus was evaluated by cold plate test measuring the latency (seconds) to pain-related behaviors (lifting or licking of the paw). Rats were daily intraperitoneally treated with 2.4 mgkg^{-1} oxaliplatin (dissolved in 5% glucose). Behavior was evaluated at day 21. Silibinin or α-tocopherol (100 mgkg^{-1}, dissolved in CMC) were per os administered acutely (single injection 30 minutes before the test; acute) or repetitively (daily for 20 days starting from the first day of oxaliplatin administration; chronic). Control animals were treated with vehicles. Each value represents the mean of 24 rats per group, performed in 2 different experimental set. *P < .01 versus CMC + glucose (control). ^P < .01 versus CMC-oxaliplatin.

paw-pressure test, significantly decreased from the control value of 69.2 ± 1.7 g (Fig 1, CMC + glucose) to 38.4 ± 2.8 g for the oxaliplatin-treated animals (CMC + oxaliplatin). Silibinin- and α-tocopherol-treated animals (100 mgkg^{-1} p.o. administered daily from day 1 to day 20) showed a significant lower responsiveness to a noxious stimulus (57.3 ± 1.6 g and 58.4 ± 2.1 g, respectively; Fig 1, chronic) in respect to the oxaliplatin group. Both molecules were ineffective when administered once, 30 minutes before test (Fig 1, acute). Moreover, oxaliplatin induced a lowered threshold to mechanical and cold stimuli which do not normally provoke pain (Figs 2A and 2B). As measured with the electronic Von Frey apparatus, control withdrawal threshold to the non-noxious mechanical stimulus was decreased in oxaliplatin treated animals from 28.2 ± 1.1 g to 14.9 ± 1.6 g (Fig 2A). Repeated silibinin administration partially modified pain threshold increasing the tolerated pressure on the anterior paw up to 19.9 ± .9 g. A comparable effect was induced by α-tocopherol (Fig 2A, chronic). The sensibility to a cold surface is depicted in Fig 2B. After oxaliplatin treatment, the licking latency decreased from 25.1 ± 1.1 seconds (CMC + glucose) to 14.2 ± .4 seconds (CMC + oxaliplatin). Both antioxidants significantly prevented cold hypersensibility (silibinin: 19.4 ± .5 seconds; α-tocopherol: 19.7 ± .2 seconds). In both Von Frey and cold plate test, silibilin and α-tocopherol acutely administered before test were ineffective. Chemotherapy-induced neuropathy impaired motor coordination as evaluated by the walking time on a rotating rod and the number of falls (Rota-rod test). Indeed, control rats were able to balance on the rotating rod for 600 seconds (Fig 3), falling down .6 ± .1 folds. On the contrary, oxaliplatin-treated animals maintained the balance for 163 ± 67 seconds only and fell down 4.9 ± .2 times. Silibinin and α-tocopherol improved the time of walking up to 415 ± 64 seconds and 409 ± 80 seconds respectively (Fig 3, chronic) but they were ineffective on the number of falls. Again, antioxidant acute treatment was ineffective (Fig 3, acute).

Aimed to evaluate the oxidative stress after the oxaliplatin administration, rat plasma samples were analyzed at the 21st day to measure TBARS and protein carbonylation. In plasma of oxaliplatin treated rats, lipid peroxidation was significantly increased as compared with control animals (CMC + glucose). The increase in TBARS induced by oxaliplatin treatment was fully prevented by silibinin and α-tocopherol repeated treatment (Fig 4A).

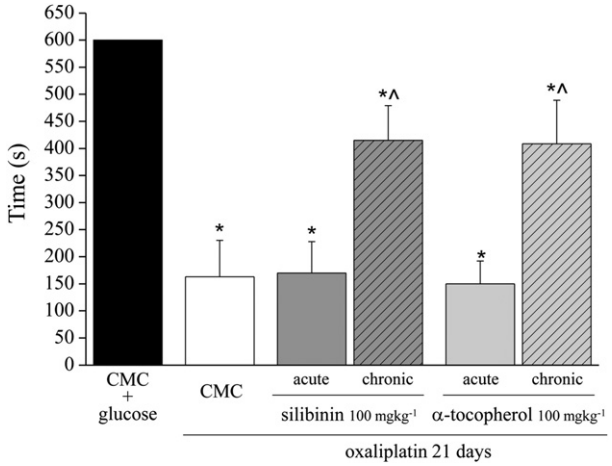

Figure 3. Motor coordination in oxaliplatin-treated rats. The integrity of the animals' motor coordination was assessed using a rota-rod apparatus. Rats were placed on a rotating rod (10 rpm) for a maximum of 10 minutes (600 seconds). The time spent in the balance was counted. Rats were daily intraperitoneally treated with 2.4 $mgkg^{-1}$ oxaliplatin (dissolved in 5% glucose). Motor coordination was evaluated at day 21. Silibinin or α-tocopherol (100 $mgkg^{-1}$, dissolved in CMC) were per os administered acutely (single injection 30 minutes before the test; acute) or repetitively (daily for 20 days starting from the first day of oxaliplatin administration; chronic). Control animals were treated with vehicles. Each value represents the mean of 24 rats per group, performed in 2 different experimental set. *P < .01 versus CMC + glucose (control). ^P < .01 versus CMC-oxaliplatin.

Carbonylated protein levels were about twice in oxaliplatin-treated rats than in control animals (Fig 4B). Protein oxidation was prevented by 81 and 58% by silibinin and α-tocopherol, respectively (Fig 4B).

The study of the oxidative damage evoked by oxaliplatin was extended to the nervous system, final target of chemotherapy toxicity. Both peripheral and central nervous system of 21 day-treated rats showed an increase in lipid peroxidation as reported in Fig 5A. Sciatic nerve TBARS levels increased in the oxaliplatin group from 38.4 ± 5.3 to 57.6 ± 3.0 μmol/mg proteins (Fig 5A, left panel) and in spinal cord from 94.7 ± 12.2 to 179.8 ± 24.3 (Fig 5A, right panel). Antioxidants exerted a preventive effect of about 80% in the sciatic nerve and 70% in the spinal cord. Moreover, oxidative stress at protein level was highlighted in both sciatic nerve and spinal cord of oxaliplatin-treated animals by a 3× increase in carbonylated level in respect to control. Silibinin and α-tocopherol inhibited protein oxidation by about 60% (Fig 5B).

Finally, DNA oxidation was evaluated in nervous tissue. In the sciatic nerve, the basal level of 8-OH-dG was 3.2 ± 2.3 pg/mL (CMC + glucose). Oxaliplatin increased this value up to 38.6 ± 8.3 pg/mL; silibinin and α-tocopherol decreased oxaliplatin-induced DNA oxidation by 48% and by 63% respectively (Fig 6, left panel). Also in the spinal cord, oxaliplatin increased 8-OH-dG from 8.7 ± 2.2 pg/mL (CMC + glucose) to 52.8 ± 4.3 pg/mL (CMC + oxaliplatin); silibinin and α-tocopherol prevented 8-OH-dG formation by 65 and 76% respectively (Fig 6, right panel).

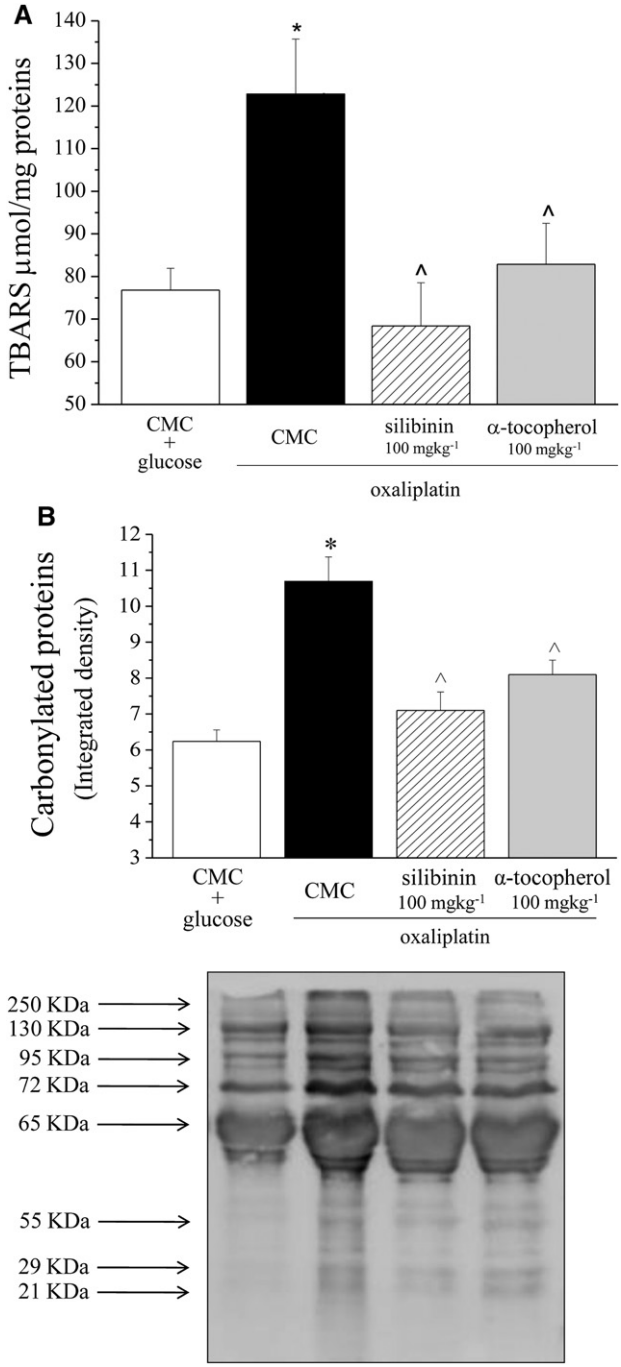

Figure 4. Plasma oxidation levels in rats treated with oxaliplatin. At day 21, plasma was collected and oxidative stress evaluated. **(A)** Lipid peroxidation was evaluated measuring TBARS levels. **(B)** Protein oxidative damage was measured quantifying carbonylated proteins by immunoblot. Densitometric analysis (top panel) and representative Western blot (lower panel) are shown. Ponceau-stained membranes were used as loading control. Silibinin or α-tocopherol (100 $mgkg^{-1}$, dissolved in CMC) were per os repetitively administered (daily for 20 days starting from the first day of oxaliplatin administration). Control animals were treated with vehicles. Each value represents the mean of 12 rats per group, performed in 2 different experimental set. *P < .01 versus CMC + glucose (control). ^P < .01 versus CMC-oxaliplatin.

Discussion

The clinical treatment of chemotherapy-induced neuropathy is based on symptomatic drugs. However, in addition to the possibility of side effects, their

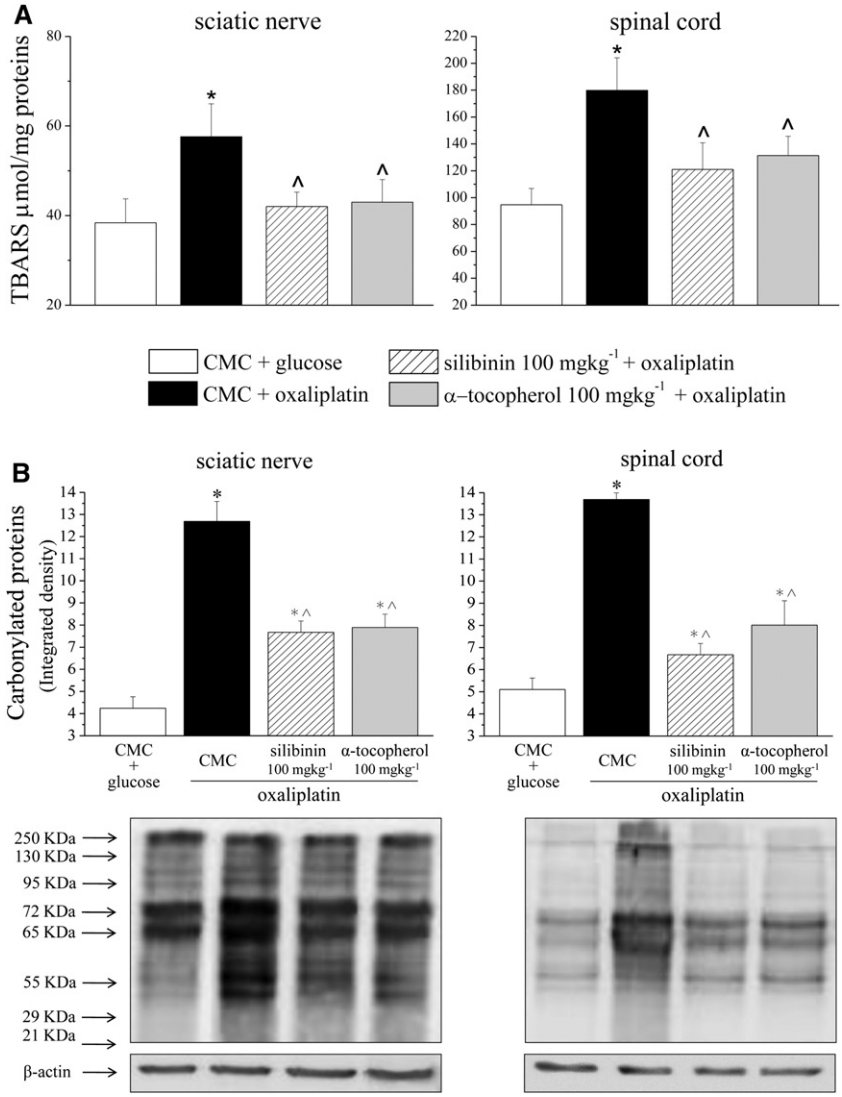

Figure 5. Nervous tissue oxidation levels in rats treated with oxaliplatin. At 21st day, the sciatic nerve and the lumbar tract of the spinal cord were explanted. Peripheral and central nervous tissues were analyzed for both lipid and protein oxidation. **(A)** TBARS levels in sciatic nerve (left panel) and in spinal cord (right panel). **(B)** Carbonylated protein in sciatic nerve (left panel) and in spinal cord (right panel). Densitometric analysis (top panel) and representative Western blot (lower panel) are shown. β-actin normalization was performed for each sample. Silibinin or α-tocopherol (100 mgkg^{1}, dissolved in CMC) were per os repetitively administered (daily for 20 days starting from the first day of oxaliplatin administration). Control animals were treated with vehicles. Each value represents the mean of 12 rats per group, performed in 2 different experimental set. *$P < .01$ versus CMC + glucose (control). ^$P < .01$ versus CMC-oxaliplatin.

effectiveness in the treatment of painful neuropathy is not yet definitely proven.[2,20,24] Therefore, the development of active disease-modifying agents is the goal of the research in this field. This issue is hampered by the insufficient knowledge of damage mechanisms.

The present data show that in the in vivo model of oxaliplatin-induced neuropathy, oxidative stress is observed at lipid, protein, and DNA levels both in plasma as well as in the nervous system. Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and it is used as an indicator of oxidative stress. TBARS are naturally occurring products of lipid peroxidation that are increased by oxidative stress.[36] Oxidative modification of proteins by ROS and other high reactive molecules such as hydroxynonenal occurs in physiologic and pathologic processes. As a consequence of the modification, carbonyl groups are introduced into protein side chains by a site-specific mechanism,[13] inducing decreased functionality. Finally, at the DNA level, hydroxylation of guanosine occurs in response to both normal metabolic processes and a variety of environmental factors. Increased levels of 8-OH-dG are associated with the aging process as well as with a number of pathological conditions including cancer, diabetes, and hypertension.[28,45]

Oxaliplatin neurotoxicity is described to be localized at dorsal root ganglia level.[47] As reported by Jacobs et al[22,23] after a single administration by i.v. injection (5 mgkg^{-1} i.v.) in nonhuman primates, the oxaliplatin

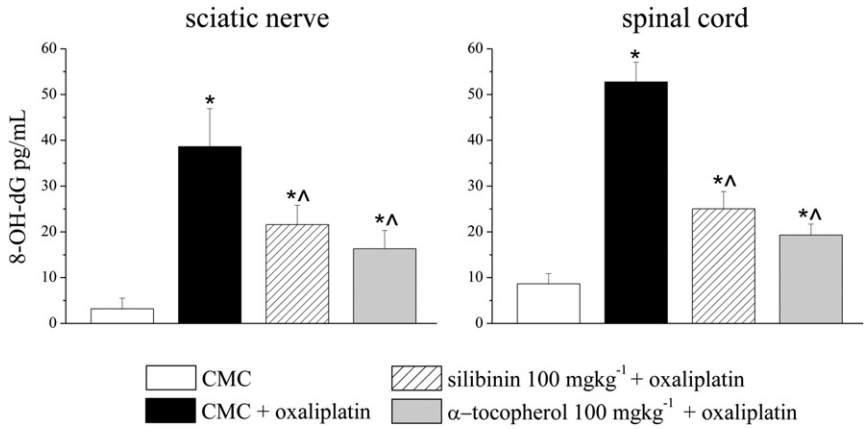

Figure 6. DNA oxidation levels in nervous tissue of oxaliplatin-treated rats. DNA was extracted from sciatic nerve and spinal cord of treated animals at day 21. DNA samples were analyzed by ELISA method to quantify 8-OH-2-dG levels. Silibinin or α-tocopherol (100 $mgkg^{-1}$, dissolved in CMC) were per os repetitively administered (daily for 20 days starting from the first day of oxaliplatin administration). Control animals were treated with vehicles. Each value represents the mean of 12 rats per group, performed in 2 different experimental sets. *$P < .01$ versus CMC + glucose (control). ^$P < .01$ versus CMC-oxaliplatin.

concentration in CSF is limited. As a matter of fact oxidative damage is here described also in the spinal cord. On the other hand, data about the oxaliplatin concentration in the central nervous system after repeated administration are lacking.

After a repeated treatment during oxaliplatin administration, antioxidant dosage (100 $mgkg^{-1}$)[19,32,49,50] of silibinin and α-tocopherol show a comparable efficacy as antihyperalgesic agents. On the contrary, acute administration of both molecules are ineffective. These results exclude a symptomatic analgesic effect of antioxidant compounds.

Moreover, a concomitant, significant decrease of the oxaliplatin-induced oxidative state is shown in plasma and in the nervous system of rats repetitively treated with silibilin and α-tocopherol.

The results lead us to hypothesize about a relationship between the preventive activity against oxidative stress and the anti-hyperalgesic properties of both molecules.

Clinically used antioxidant agents partially reduce oxaliplatin-induced toxicity without a precise explanation for their limited, beneficial effects.[7,29,38] It remains to be seen if their therapeutic limited effectiveness is due to the scarce activity of the presently available substances or to the complexity of oxaliplatin neurotoxicity where oxidative stress could be only an epiphenomenon.

On the other hand, the mechanism by which oxaliplatin provokes ROS increase is not completely established and it could be due to a characteristic cell damage. A mitochondrial alteration has been suggested as a mechanism of oxaliplatin-mediated oxidation.[51] Moreover, dynamin-related protein 1, a protein that catalyzes the process of mitochondrial fission with the consequent ROS production, is involved in chemotherapy-induced neuropathy in rats.[16] By contrast, a low relevance of ROS production from inflammatory cells could be hypothesized since another side effect related to prolonged oxaliplatin use is a nonmyelosuppressive hematologic toxicity, which includes hemolysis,[18] thrombocytopenia,[5] and pancytopenia.[48]

It is of fundamental importance that, to be clinically useful, the antineuropathic agents must reduce the neurotoxic effect of the chemotherapeutic agent maintaining its full anti-tumor efficacy. In this light, silibinin shows a cancer chemopreventive role in both in vitro[10,21] and in vivo[40,46] models. It modulates the imbalance between cell survival and apoptosis through interference with the expressions of cell cycle regulators and proteins involved in apoptosis.[26] In addition, an anti-metastatic activity for silibinin has been also described.[11,21] A recent report of the USA National Toxicology Program[33] describes the results of repeated treatments of rats with sylibum marianum extract (containing 65% sylimarin, therefore 30–40% silibinin).[1] No toxic effects have been shown after daily per os administration of 2,500 $mgkg^{-1}$ extract for 2 years. Moreover, there is no evidence of carcinogenic activity whereas the incidences of mammary gland neoplasms and hepatocellular neoplasms are decreased. In this view, silibinin should not interfere with the anticancer property of oxaliplatin.[41]

The present data highlight the anti-neuropathic properties of silibinin which, together with its protective effects in hepatic diseases[15,30,31,44] as well as in neurodegenerative disorders,[31] suggest its clinical applicability as an adjuvant in cancer patients to prevent or reduce chemotherapy-induced toxicity. The protective effects and the anticancer activity confer to silibinin a ubiquitous profile that differentiates this molecule from other antioxidant drugs clinically used to treat oxaliplatin-induced neuropathic pain.

Moreover, according to our results, oxidative stress is an important target in oxaliplatin-dependent neuropathic pain and allows us to identify a mechanism of oxaliplatin-induced neurotoxicity. Finally, the research of fully active antioxidant compounds remains an attractive therapeutic perspective to treat neuropathy through neurorestorative effects.

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